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ABSTRACT

The eye is an ideal target for exploiting the potential of human induced pluripotent stem cell (hiPSC) technology in order to understand disease pathways and explore novel therapeutic strategies for inherited retinal disease. The aim of this article is to map the pathway from state-of-the art laboratory-based discoveries to realising the translational potential of this emerging technique. We describe the relevance and routes to establishing hiPSCs in selected models of human retinal disease. Additionally, we define pathways for applying hiPSC technology in treating currently incurable, progressive and blinding retinal disease.

Contents

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1. Introduction

Stem cells have the ability to self-renew and the capacity to differentiate into other more specialised cell types. Cell potency describes the general ability of a cell to differentiate into other cells. During human development, one of the first stem cells to arise is the human embryonic stem cell (hESC) which can be found in the inner cell mass of the developing embryo. The embryonic stem cell has the potential to differentiate into cells from the three germ layers: endoderm, mesoderm and neuroectoderm. Consequently the hESC is described as pluripotent. Other stem cells emerge later during development which display a progressively more restricted phenotypic range and can be considered tissue or organ-specific. In adults, endogenous tissue-specific stem cells are multipotent with a more restricted differentiation repertoire normally confined to those cells of the tissue of origin.

James Thomson reported successful isolation and maintenance of hESCs in 1998 (Thomson et al., 1998). HESCs have an infinite proliferation capacity and offered the opportunity to provide a virtually unlimited supply of human cells for clinical translation research. However, ethical concerns regarding the derivation of cells from the inner cell mass of developing blastocysts led to a drive to investigate alternative methods of deriving pluripotent stem cells (Ramalho-Santos, 2011). The methodology that eventually emerged to produce pluripotent stem cells was developed on the background of several breakthroughs in cell biology. Sir John Gurdon first demonstrated the cloning of an adult frog from the transfer of adult intestinal cells into an enucleated *Xenopus laevis* ovum (Gurdon, 1962). This illustrated that adult cell fate was not restricted and that under appropriate conditions differentiated somatic cells could be made pluripotent. Similar nuclear transfer into mammalian cells was more problematic due to the smaller size of mammalian eggs. Although successful mammalian nuclear transfer was later demonstrated using embryonic cells nuclear transfer cloned animals did not develop from differentiated cell nuclei (Cheong et al., 1993; Prather et al., 1989). Wilmut et al. demonstrated that these difficulties could be overcome by nuclear transfer into early embryos. Using this technique an adult sheep was cloned by nuclear transfer from an adult sheep mammary gland cell into a day 9 embryo (Wilmut et al., 1997). This demonstrated that specific factors exogenously expressed by developing embryos can return somatic cells to a pluripotent state. In 2006, Shinya Yamanaka isolated four transcription factors that when expressed exogenously induced the formation of pluripotent cells from somatic cells. This was first confirmed using murine and subsequently human somatic cells (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The process of generating pluripotent cells from somatic cells was termed "reprogramming" and the resultant cells were called induced pluripotent stem cells (iPSCs). iPSCs shared properties with hESCs including the ability to self-renew and to be differentiated into the three germ layers.

The clinical translation of basic scientific discoveries to treatments has been made a priority of national funding bodies worldwide (McLellan, 2003; MRC, 2013). Ophthalmic research has been at the forefront of the drive for clinical translation. The eye has several properties that are advantageous as an organ suitable for regenerative approaches including relative ease of accessibility, immune privilege and relative isolation from other body systems. HiPSC technology was developed relatively recently on the foundation research in several fields of basic science, the technology is nearing the point of full clinical translation. Recently, hiPSC derived retinal pigment epithelium (hiPSC-RPE) have been approved for use in patient safety trials for the treatment of macular degeneration (Cyranoski, 2013). This article aims to provide a background into the current state of research in this rapidly evolving field with a focus on the cells of the outer retina. We provide a summary for planning hiPSC studies, describing hurdles to clinical translation as well as highlighting future directions of research using hiPSC-derived retinal cells.

2. Basic principles of human somatic cell reprogramming

Complete reprogramming involves the replacement of the tissue specific donor cell transcription factors with those that will induce pluripotency. Additionally, reprogramming requires the epigenetic stabilisation of the new machinery. The original reprogramming strategies have provided valuable insight into the mechanisms involved. A variety of different approaches have now been established to achieve reprogramming since the original procedures described by Yamanaka and Thomson. However, as our knowledge has progressed, the criteria for an ideal protocol have become clearer. The characteristics of an ideal protocol include:

1. Free from Variation
2. Free from Integration
3. Efficient
4. Fast
5. Frugal

2.1. Protocols

In the original reprogramming experiments two sets of transcription factors were identified concurrently but independently by Yamanaka and colleagues in Kyoto, Japan (Takahashi et al., 2007) and Thomson in Madison, Wisconsin, USA (Yu et al., 2007) (Table 1). Both groups used OCT4 and SOX2, but they included variations in other factors. Yamanaka used KLF4 and c-MYC whereas Thomson used NANOG and LIN28. The groups both used retroviral vectors, but whilst Yamanaka and colleagues used the pMXs plasmid back-bone derived from Moloney murine leukaemia virus, Thomson used lentiviral vectors. Lentiviral vectors have the advantage of being able to integrate in non-dividing cells. Lentiviral mediated insertion is still the most frequently used

Table 1
Transcription factors commonly used in somatic cellular reprogramming.

Transcription factors for reprogramming	Function
KLF4	A member of the Krüppel-like family of zinc finger transcription factors that is involved in cell proliferation, differentiation and survival.
C-MYC	This transcription factor controls the expression of a multitude of target genes. It is involved in cell proliferation, apoptosis, and self-renewal. It is activated by other pathways including the WNT, epidermal growth factor and sonic hedgehog pathways. C-MYC is also a potent oncogene implicated in several cancers.
OCT4	This transcription factor forms a complex with SOX2 and DNA. It plays a crucial role in early embryonic development and is necessary for the maintenance of embryonic stem cell pluripotency.
SOX2	SOX2 is important in early development and is required to maintain self-renewal of undifferentiated embryonic and neural stem cells.
NANOG	NANOG is expressed in undifferentiated cells, including foetal gonadal organs, the inner cell mass and in embryonic stem cells. NANOG prevents cells in the inner cell mass from differentiating into extra-embryonic, endodermal and trophoectodermal lineages.
LIN28	Protein which binds microRNA and enhances the translation of insulin like growth factor 2. LIN28 plays a role in self-renewal of cells.

technique as protocols are now both optimised and reliable with commercially available vectors. We discuss the potential merits of other protocols using relevant paradigms (Table 2).

2.2. Variation: fully versus partially reprogrammed

Criteria for reprogramming have varied over time. There is considerable disagreement as to the relative importance of these two mechanisms. In some rare circumstances single transcription factors alone are able to change the fate of cells from one phenotype to another, but in these cases the change is within the same germ layer (Davis et al., 1989). In contrast, when the change is between germ layers, as is the case during production of hiPSCs, several transcription factors are still required (Takahashi et al., 2007; Yu et al., 2007).

With the original reprogramming protocols it was essential that all factors were not only integrated within the somatic cell genome but also expressed within a relatively narrow stochastic window. However, transcription factor expression varied due to differences in the epigenetic state of the insertion sites. This caused marked variation in the daughter cells. Consequently, only 1 in 10,000 cells became pluripotent (Hanna et al., 2009). Consequently, many colonies had to be maintained in order to identify cells with the appropriate pluripotent phenotype.

Those cells in which factors are expressed beyond the stochastic window of fully reprogrammed cells are termed partially reprogrammed. These cells hamper pluripotent cell culture by rapidly colonising plates (Mikkelsen et al., 2008). Partially reprogrammed cell numbers can however be reduced by feeder free derivation and culture (Chan et al., 2009). The combination of cell variation and partially reprogrammed cells prolong pluripotent cell colony isolation and therefore lead to increased costs for media and labour.

2.3. Integration versus integration free

There are now several procedures that meet the second requirement in full. A stably integrated drug inducible system has provided important opportunities to analyse factors that influence the speed and efficiency of fate change (Wernig et al., 2008). Notably, colonies were only seen if transgene expression was induced for 9 days or more, regardless of the duration of transgene expression. This pattern is similar to that found following primary infection by retroviral vectors (Brambrink et al., 2008). Surprisingly, after transgene expression for 9 days the number of colonies continued to increase until day 25, which is 15 days after withdrawal of the drug inducement (Wernig et al., 2008).

The use of integrating vectors can be avoided in several ways. Firstly, non-integrating viral vectors have been used successfully, including adenovirus, adeno-associated virus and Sendai virus. However, these vectors are still biologically active and viruses may remain in cells for several passages.

Reprogramming methodologies which are non-integrative and not biologically active have become available. An example are DNA plasmids which have been used successfully (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Okita et al., 2008; Stadtfeld et al., 2008; Fusaki et al., 2009; Zhou and Freed, 2009; Si-Tayeb et al., 2010; Nakanishi and Otsu, 2012). Plasmids are introduced by transfection. Although these do not carry any mechanism to promote integration, there is a theoretical risk that this may occur through the activity of DNA repair mechanisms making it necessary to ensure that DNA has not been incorporated into chromosomes before such cells can be used in therapy. Additionally, excisable systems have been developed after integration (Kaji et al., 2009; Woltjen et al., 2009; Somers et al., 2010) as well as DNA-free reprogramming methods such as mRNA, protein and small molecules (Kim et al., 2009; Zhou et al., 2009; Warren et al., 2010; Lin et al., 2009).

2.4. Efficiency

The efficiency of reprogramming is influenced by chromatin organisation in the treated cell and the speed of the cell cycle.

Table 2
Advantages and disadvantages of available reprogramming methodologies.

Technique	Integrative	Non-integrative	
		Biologically active	Biologically inactive
Methods	Lentiviral, Moloney murine leukaemia virus	Sendai virus, adeno-virus	RNA, proteins, minicircle, episomal
Advantages	Stable, Efficient vectors, relatively cheap	Integration free	Integration free Not biologically active Likely to have less variation
Disadvantages	Integrated leading to copy number variations, Genetic footprint	Adenovirus is slow and inefficient. Biologically active viruses remain in cells despite several passages with potential for immune reaction if used for cell replacement	These are all slow and inefficient except RNA and often requiring multiple transfections with the same cells. Labour intensive and therefore costly at present

Optimisation of these characteristics can be of considerable practical value. Small molecules have also been used to promote the effect of transcription factors by modifying chromatin in the donor cells. Examples include 5-aza-deoxycytidine which promotes demethylation of DNA (Wernig et al., 2008) and valproic acid which inhibits incorporation of a histone deacetylase (HDAC) (Huangfu et al., 2008). Similar beneficial effects were seen when the powerful transactivator from *MyoD*, the gene that has a dominant role in inducing muscle formation, was incorporated alongside the *Oct4* coding sequences (Hirai et al., 2012). Incorporation of ascorbic acid in the culture medium had an unexpected effect upon the reprogramming process (Esteban et al., 2010). Further analysis showed that this effect reflected the ability of ascorbic acid to enhance the activity of two H3K36 demethylases (Jhdm1a/1b). This effect resulted from acceleration of cell cycle progression and suppression of senescence by Jhdm1b, which also cooperates with OCT4 to activate the microRNA cluster 302/367 an integral component of the pluripotency network (Wang et al., 2011). Ascorbic acid was also shown to reduce hypermethylation of the imprinted gene cluster *Dlk1-dio3* by preventing the binding of the DNA methyltransferase DNMT3A. As a result of these effects, mouse iPSCs produced from mature B-cells in the presence of ascorbic acid acquired the ability to form offspring by tetraploid embryo complementation which was derived exclusively from iPSCs (Stadtfeld et al., 2012). Taken together these observations emphasise the importance of the epigenetic environment in determining the efficiency and accuracy of cellular reprogramming.

A number of observations point to the importance of cell division in the process of reprogramming. A beneficial effect of the incorporation of c-MYC in the reprogramming recipe was interpreted as reflecting an effect on the cell cycle. Similar effects were seen during a detailed analysis of the effects of overexpression of a different oncogene, *LIN28* or of the inhibition of the p53 pathway. Both of these changes increased the speed of reprogramming in direct proportion to the increase in cell division (Hanna et al., 2009). The addition of NANOG to the regime establishes enhanced reprogramming through a network of other transcriptional factors once factors such as OCT4 have been instituted (Hanna et al., 2009).

Two other means of enhancing the efficiency of reprogramming have been described. Serum starvation before treatment led to an increase in the effectiveness of standard reprogramming in both skin fibroblasts and adipose stem cells (Chen et al., 2012). The protocol may have synchronised cells in G0/G1 such that when released they passed through the next mitosis as the transgenes first expressed the reprogramming proteins. Selection of fibroblasts that expressed the embryonic antigen SSEA-3 before treatment also led to increased efficiency in reprogramming (Byrne et al., 2009). No iPSCs were obtained from those cells that did not express SSEA-3. It seems very likely that other means of enhancing reprogramming to a pluripotent state remain to be discovered. For instance, it has been shown that it is possible to remove C-MYC from the regime with the addition of a microRNA cluster (Judson et al., 2009). This may be of relevance when creating lines for clinical use to reduce the oncogenic risk.

Although significant progress has been made in reprogramming technologies the ideal protocol is still not currently available and the protocol selected is a balance between each of the paradigms described. The choice of protocol is dependent on the final aims of the reprogramming. If reprogramming is used to study mechanisms of reprogramming alone then a mouse cell can be used with a cheaper and more robust and integrated methodology. However, if replicating human physiology or clinical cell replacement is required then a biologically inactive, integration free method would be more suitable using human cells preferably not containing any mutagenic transcription factors.

2.5. Donor cell

A further factor to consider is the donor cell type. To date most reprogramming has been performed through donor fibroblasts. These cells are easy to obtain and robust in culture. However, they are not the most efficient cells to reprogram. Additionally, the punch biopsy procedure is invasive and not suitable for use in young children. A number of other cell types have also been used for reprogramming including keratinocytes (Aasen et al., 2008), melanocytes (Utikal et al., 2009) and cord blood (Giorgetti et al., 2009). Recently, a number of groups used constituents of whole blood including circulating T-cells and endothelial progenitor cells obtained by venepuncture (Howden et al., 2011; Geti et al., 2012; Phillips et al., 2012). This offers the obvious advantage of using samples from routine testing that can be easily transported. Efficiency and ease of reprogramming varies between donor cell types. This is thought to be due to variations in endogenous expression of transcription factors (Kim et al., 2010) (Fig. 1).

2.6. Selection of hiPSCs

Use of hiPSCs is complicated by the derivation of partially reprogrammed cells alongside fully reprogrammed ones. In these circumstances it is essential to carry out a rigorous assessment of cell lines prior to use in order to confirm that reprogramming is complete. The main tools to characterise hiPSCs are summarised in Table 3 and Fig. 2.

Morphologically stem cell colonies have a high rate of cell division. Cells are tightly packed with large nuclei. Cell surface markers include SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 (Takahashi et al., 2007). More recently use of live-cell staining techniques has become possible for markers such as TRA-1-60 to assist in the isolation of pluripotent colonies. Routine reverse transcription and polymerase chain reaction (rtPCR) can be used to confirm expression of factors associated with pluripotency including *NANOG, OCT4, SOX2, REX1* and *hTERT* (Takahashi et al., 2007). Pluripotency can be confirmed by a number of methods. Transplanting hiPSCs into immunodeficient mice to show teratoma

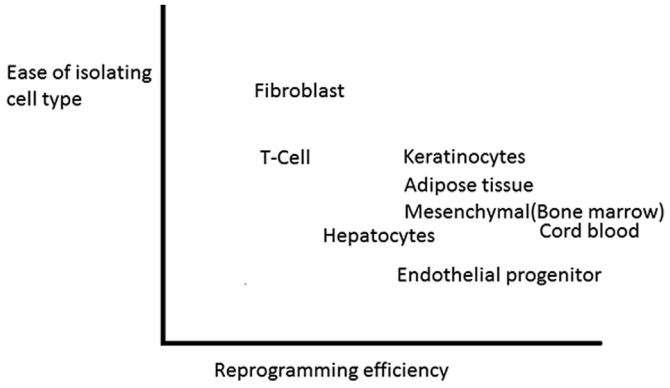

Fig. 1. Comparison of relative ease of reprogramming efficiency to ease of isolation of donor cell type. Fibroblasts are the most commonly used donor cell. They are amongst the easiest to prepare for reprogramming as they are relatively easy to access, propagate as a pure population and maintain. Cells such as mesenchymal cells and hepatocytes require more invasive procedures for extraction. Endothelial progenitor cells and keratinocytes require isolation from blood and skin respectively. Additionally they require specialised media to propagate effectively (Aasen et al., 2008; Eminli et al., 2009; Geti et al., 2012).

Table 3
Methods commonly used to validate hiPSCs.

Characteristic	Expected results
Morphology	Flat colonies High nuclear to cytoplasmic ratio Small, round shape
Cellular markers	Nanog, Sox2, Oct4
Cell surface markers	SSEA-3, TRA-1-60, TRA-1-81
rtPCR/ICC	*NANOG, OCT4, SOX2, REX1* and *hTERT*
Bisulphite sequence analysis	Promoter methylation status of the endogenous pluripotency genes
Quantitative PCR (silencing of the exogenous reprogramming factors)	*NANOG, OCT4, SOX2, C-MYC, KLF4*
Karyotyping	Chromosomal stability
Teratoma formation/embryoid body formation	Standard teratoma formation to test pluripotency is performed in immunodeficient mice, injection routes are sub-renal capsule, subcutaneous or intramuscular. Differentiation capacity of hiPSCs to three germ-layers; endoderm, mesoderm and neuroectoderm

formation still remains the gold standard (Jaenisch and Young, 2008). A cheaper alternative is to show embryoid body formation *in vitro* in order to show functional pluripotency (Itskovitz-Eldor et al., 2000; Kurosawa, 2007). Using this methodology hiPSCs are differentiated using standardised protocols to develop cells from different germ layers. Differentiation can be confirmed by immunostaining and RNA expression (Fig. 2).

More recently, molecular assessments have been made. This may involve monitoring gene expression by gene array to assess the function of either selected gene populations or the entire genome. As reprogramming depends upon major changes in the epigenome, direct measurements have been made of the epigenetic status of the cell during and following treatment. These include assessment of the promoter regions of key pluripotency genes or genome wide assessment of DNA methylation, histone methylation and acetylation. By combining a number of these observations several laboratories have developed schemes for the selection of those lines that have been reprogrammed accurately (Bock et al., 2011; Chan et al., 2009). Others have placed great emphasis on correct regulation of imprinted genes (Lujan and Wernig, 2010). Most recently, a systematic survey of cell surface glycoproteins has been initiated with the hope that this may define cell surface patterns that are cell type specific (Gundry et al., 2012). Consequently, the landscape of validation is constantly evolving.

In practice it is likely that most research laboratories will be satisfied with a relatively modest survey of markers and function. It is greatly hoped that in the near future methods for hiPSC production will become sufficiently reproducible and accurate that extensive assessment will no longer be required unless the cells are to be used for cell therapy.

3. Retinal differentiation

During human embryogenesis the inner cell mass of the blastocyst forms the epiblast. The epiblast divides into the embryonic

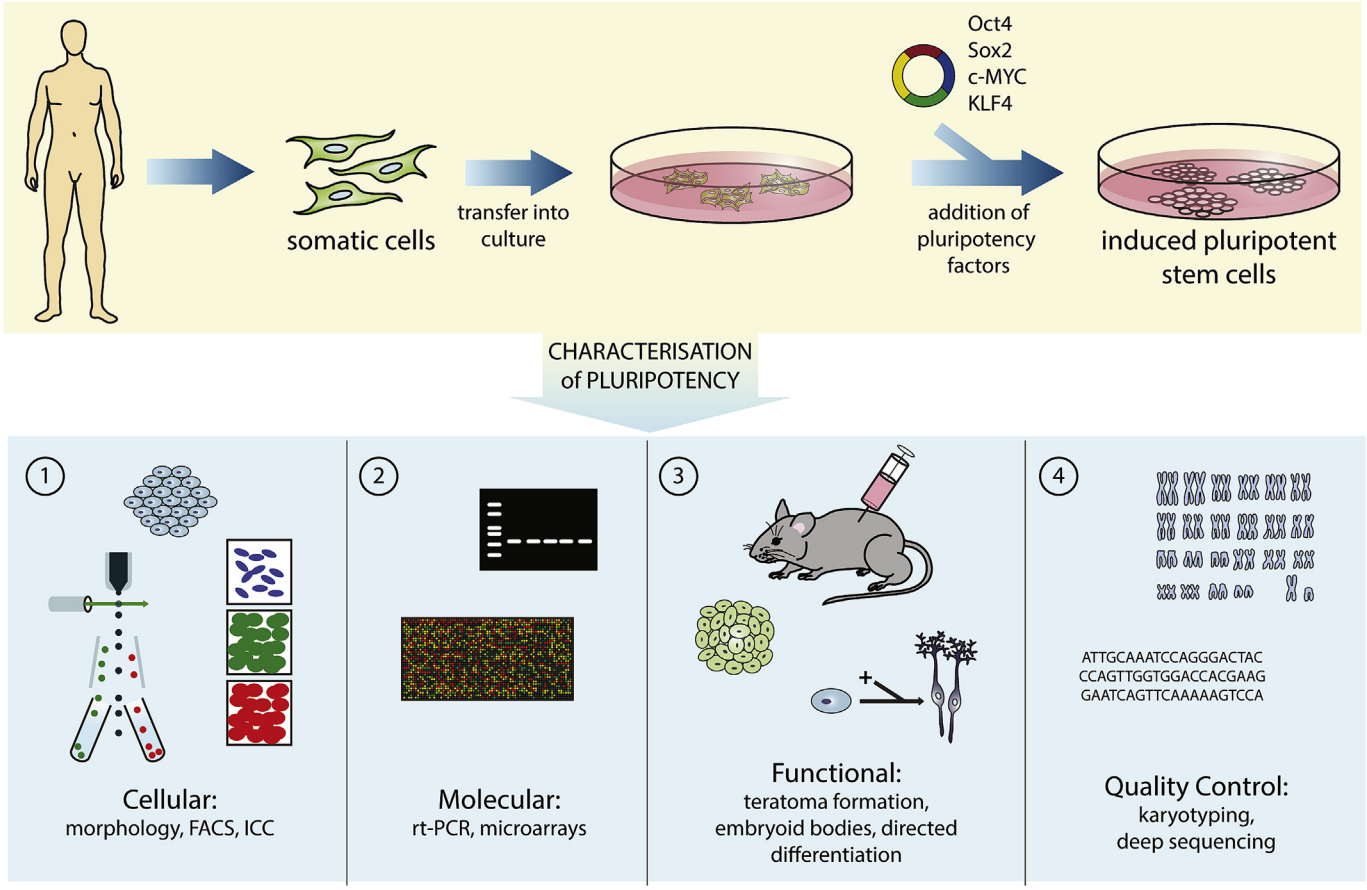

Fig. 2. Characterisation of pluripotency. In order to reprogram human somatic cells the cells are first isolated and cultured *in vitro* for expansion. Various methods have been employed to introduce reprogramming transcription factors into cells. These are summarised in Table 2. Only a small proportion of the donor cells will form pluripotent colonies which have hESC-like properties. Traditionally a range of techniques has been used to identify pluripotent cells. The common techniques are illustrated here and can be divided into cellular, molecular, functional and quality control streams for validation of pluripotency.

germ layers (ectoderm, mesoderm and endoderm). Development of the neuroectoderm occurs from the ectodermal germ layer. The retina arises through mid-ventral and rostral patterning and results in the formation of the optic grooves from the rostral diencephalon, part of the primitive forebrain. Later, the optic grooves protrude to form optic vesicles. At a cellular level, hESCs undergo stepwise differentiation through neural and retinal progenitor stages before differentiating to either RPE or neuroretinal cell types (Fig. 3).

In vitro retinal differentiation protocols for hiPSCs have been developed on the groundwork of hESC studies. HESCs have been shown to recapitulate the differentiation of retinal cells *in vivo* (Klimanskaya et al., 2004; Lamba et al., 2006; Meyer et al., 2009; Osakada et al., 2008). *In vitro* retinal differentiation protocols for hiPSCs are broadly divided into two main categories. Firstly, default differentiation, in which cells are cultured in the absence of extrinsic growth factors. Secondly, directed differentiation protocols, in which differentiation requires the addition of extrinsic transcription factors, proteins and small molecules. Currently, no differentiation protocol is absolutely efficient in its production of retinal cells. Therefore the choice of differentiation protocol depends on the resources available, the aims of the research and the final purity of cell population required.

3.1. Default differentiation

In an incompletely understood mechanism, human pluripotent cells differentiate to neural lineages, including those of the retina, in the absence of exogenous factors (Munoz-Sanjuan and Brivanlou, 2002). This is often known as the default model of pluripotent cell differentiation. There is increasing evidence from mammalian ES cell studies that this results from a cell intrinsic mechanism (Kamiya et al., 2011; Smukler et al., 2006).

Fibroblast growth factor, activin and Nodal have been shown to be key regulators of pluripotency in human pluripotent cells (Vallier et al., 2005). Removing basic FGF from pluripotent cell maintenance media initiates differentiation (Reubinoff et al., 2001). Neuroectodermal induction requires suppression of other differentiation pathways including those to mesodermal and endodermal cell fates. In default differentiation this is thought to result from the suppression of bone morphogenetic protein (Tropepe et al., 2001). Differentiating cells appear to follow embryologic differentiation pathways in which the pluripotent cells attain a retinal progenitor stage before expressing more mature retinal cell markers including those for RPE and neuroretina (Vugler et al., 2008). RPE cells can be identified by melanin pigmentation within four weeks of differentiation in default culture conditions (Carr et al., 2009). Isolation, culture and expansion of patches of pigmented polygonal cells can be used to derive confluent monolayers of RPE. This is perhaps the simplest of protocols for differentiation of RPE and requires little specialised media or intervention. This also means that large volumes of hiPSCs can be grown. However, limitations include the relative inefficiency of differentiation resulting in a mixture of cell types potentially wasting expensive media. Additionally, some lines may not have an intrinsic capacity for retinal differentiation which may be dependent on endogenous expression of retinal differentiation genes (Mellough et al., 2012). This is supported by analysis of the transcriptome from RPE cells following differentiation shows marked variation in RNA expression. This suggests that inherent differences between cell lines could lead to differences in retinal differentiation (Liao et al., 2010; Strunnikova et al., 2010).

3.2. Directed differentiation

Directed differentiation is a method that guides cell differentiation with the addition of exogenous factors. Patterning occurs rapidly during embryogenesis. Germ layer cell fate is determined as early as the end of the first week. Table 4 summarises the transcription factors, proteins and small molecules used in directed differentiation to retina.

It is has already been demonstrated that pluripotent cells pass through an anterior neuroectoderm like stage when recapitulating retinal differentiation *in vitro* (Meyer et al., 2009) (Fig. 3). A number of published protocols have attempted to increase the efficiency of differentiation to neuroectoderm from pluripotent cells. Chambers et al. showed that neuralisation increased to over 90% with inhibition of the SMAD signalling pathway using the protein NOGGIN and the small molecule SB431542. This contrasts with less than 10%

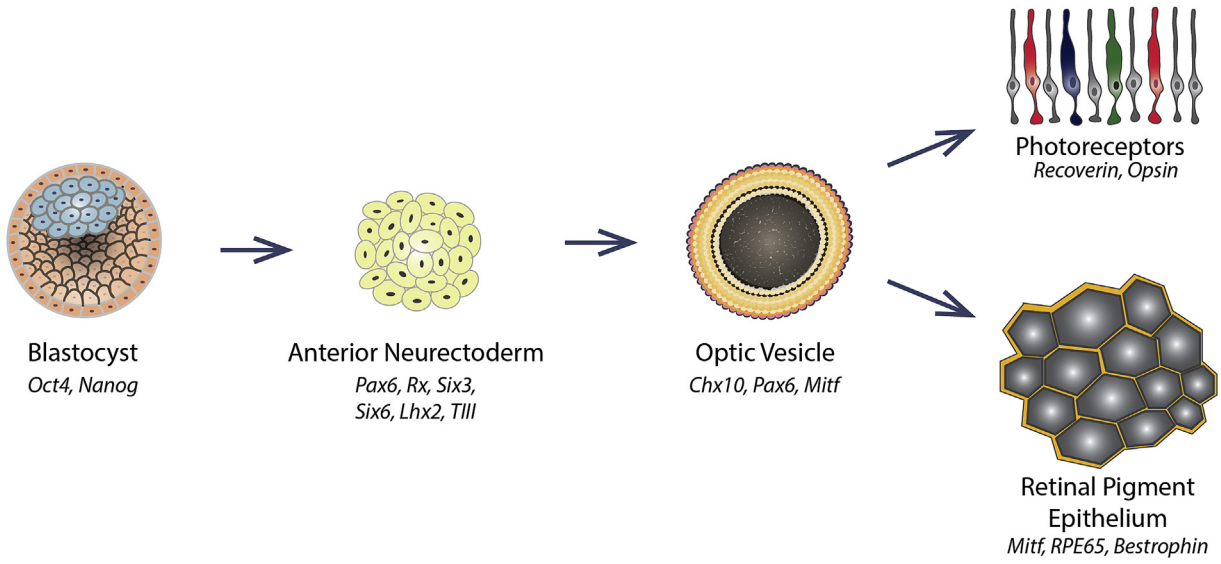

Fig. 3. Retinal differentiation of pluripotent stem cells. Pluripotent cells differentiate through neural and retinal progenitor stages. MITF is a marker for retinal progenitor cells. Differentiation to RPE requires the continued expression of MITF without up-regulation of CHX10. Alternatively differentiation to neuroretina requires the enhanced expression CHX10 with suppression of MITF. Expression of these key regulators is modified by FGF, TGF-β and WNT signalling (Meyer et al., 2009; Nguyen and Arnheiter, 2000).

Table 4
Summary of commonly used transcription factors, proteins and small molecules in retinal directed differentiation protocols.

Factor	Normal function
Noggin	Noggin is a protein which prevents binding of TGF-β ligands to their receptors. Noggin plays a key role in neural induction by inhibiting bone morphogenetic protein.
SB431542	Small molecule used to selectively inhibit the TGF-β1 receptor. Initially used to suppress differentiation of osteosarcoma cells.
Lefty	Important in left-right patterning. Lefty is an antagonist of Nodal signalling.
DKK-1 (Dickopf-related protein 1)	A protein which is an inhibitor of the WNT signalling pathway.
Sonic Hedgehog	A protein important in ventral/rostral patterning of the brain and limbs.
Nicotinamide	An amide of vitamin B3/niacin. Although the mechanism of action is incompletely understood there is increasing evidence to show that nicotinamide promotes neuralisation and reduces apoptosis.
Activin A	A member of the TGF-beta superfamily. Activin A interacts with the Nodal and WNT systems and has a wide range of biological activities, including mesoderm induction, neural cell differentiation, bone remodelling, haematopoiesis and roles in reproductive physiology.
IWR1-Endo	Small molecule inhibitor of WNT signalling.
CHIR99021	Selective inhibitor of glycogen synthase kinase 3.

neuralisation efficiency in the absence of SMAD inhibition (Chambers et al., 2009). Similar results have been found by a number of other groups who have showed dual SMAD inhibition was greater than inhibition of a single SMAD pathway (Lee et al., 2007; Smith et al., 2008) (Fig. 4). Osakada et al. also investigated the use of the small molecules in SMAD inhibition. It was suggested these non-antigenic small molecules would be advantageous in the derivation of cells used for cell replacement therapies. Casein kinase 1 when used in combination with SB431542 showed a similar efficiency to LEFTY and DKK-1 (Osakada et al., 2008). Taken together these studies demonstrate the importance of modifying Nodal, WNT, BMP and TGF-β in early neuroectodermal patterning.

The composition of media may also play a part in differentiation. B27 (Lu et al., 2009) or N2 (Meyer et al., 2011) supplementation has recently been shown to be important in accelerating neural and retinal differentiation (Mellough et al., 2012). Traditionally, *in vitro* differentiation has been performed at atmospheric oxygen concentration (20%). This is markedly different from *in vivo* oxygen levels in tissues which are likely to be in the range 2–5% (Erecinska and Silver, 2001). Hypoxia is thought to activate the phosphatidylinositol pathway and lead to downstream up-regulation of S6, HIF, cyclin and BCL which all play a part in cell growth and proliferation. A 3% oxygen concentration increases the percentage of viable cells at the neuroectodermal stage (Stacpoole et al., 2011).

3.2.1. Directed differentiation to RPE

After the primitive anterior neuroectodermal stage of differentiation, cells that ultimately have a retinal cell phenotype undergo a critical switching. Cells which differentiate to RPE increase MITF expression whilst those that differentiate to neuroretina upregulate CHX10 expression and suppress MITF (Fig. 3).

Addition of activin-A has been found to increase MITF expression 8.3 fold (±2.5) and also convert a subset of optic vesicle like structures to an RPE fate (Meyer et al., 2011 Stem Cells) (Fig. 3). Idelson et al. (2009) found that the addition of nicotinamide to activin-A resulted in a further increase of RPE differentiation from hESCs. The exact mechanism of action of nicotinamide was not investigated. Nicotinamide has subsequently been used at both initial and late stages in protocols to differentiate hiPSCs successfully (Kokkinaki et al., 2011; Ukrohne et al., 2012). It is thought to have an anti-apoptotic promoting neuronal cell viability (Cimadamore et al., 2009). Recently, a combination of factors which had been used in previous protocols demonstrated a rapid differentiation of hESCs and hiPSCs to RPE. Buchholz et al. (2013) used a combination of DKK-1, Noggin, nicotinamide and insulin-like growth factor, with the later addition of FGF and activin-A to produce expandable pigmented RPE population which was identified as early as day 14.

RPE forms easily identifiable pigmented, polygonal colonies. The differentiated RPE can be mechanically dissociated before replating for expansion (Lu et al., 2009). Expansion of cells has been described using various concentrations of foetal calf serum (2–10%) (Sonoda et al., 2009). A serum free method has been described using media containing basic FGF and EGF supplemented with heparin (Gamm et al., 2008; Lu et al., 2009). RPE appears to undergo rapid senescence with passaging. By passage 3 hiPSC-RPE were found to have telomeres which were 60% shorter than the original hiPSCs. Additionally, cells were noted to have marked DNA damage at later passages (Kokkinaki et al., 2011). As well as being propagatable, hiPSC-RPE can also be banked for later use (Lu et al., 2009; Nakano et al., 2012).

A definition of hiPSC-RPE is relevant for both modelling and transplantation studies in order to ensure that there is similarity to the *in vivo* cell phenotype and also to allow comparison between readouts from derived cells lines. Although a standard definition of RPE has yet to be agreed, a range of characteristics can now be

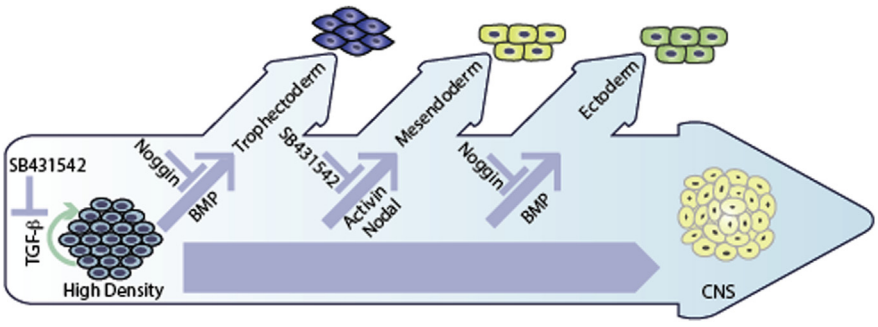

Fig. 4. Summary of the effects of dual SMAD inhibition with NOGGIN and SB431542. Dual SMAD inhibition is proposed to interfere with TGF-β and Nanog interactions and consequently drive differentiation. Neuralisation is promoted by the suppression of alternative potential cell fates. This results in the formation of a primitive anterior neuroectoderm.
Adapted from Chambers et al., 2009

reliably assessed in order to describe the RPE phenotype. These include morphological, molecular and functional features (Table 5). Certain markers continue to be expressed in hESC/hiPSC-RPE despite prolonged culture, including PAX6. RPE specific markers include those for phagocytosis such as MERTK and the basal marker, BEST1. Apical membrane associated markers include Na^+/K^+ATPase and Ezrin. Pigmentation markers include tyrosinase and secreted growth factors such as pigment epithelium derived factor (PEDF). Visual cycle markers include RPE65, LRAT and CRALBP (Bharti et al., 2011).

3.2.2. Directed differentiation to neuroretina

Several methods of achieving highly enriched populations of hiPSC-derived neuroretinal progenitor progenitor cells (NRPCs) have been described that utilise neuroretinal fate-biasing culture media (Hirami et al., 2009; Jin et al., 2011; Lamba et al., 2010; Mellough et al., 2012; Osakada et al., 2009). Both hESC- and hiPSC-derived neuroretinal progenitor cells (NRPCs) differentiate in a similar manner, mimicking the order and time course of normal retinogenesis (Meyer et al., 2011; Meyer et al., 2009; Phillips et al., 2012) (Table 6). Lamba et al. (2006) showed that hESCs could be directed to a neuroretinal cell fate with the addition of NOGGIN, DKK-1 and IGF-1. Cells were then maintained in basic FGF for 3 weeks. The protocol led to a 75–165 fold increase in the expression of eye field transcription factors *RX, PAX6, SIX3* and *LHX2* when compared with cells grown in media without additional factors. However, the protocol failed to produce mature photoreceptors with high efficiency with less than 0.01% of cells expressing *S-OPSIN* and *RHODOPSIN* (Lamba et al., 2006).

Guided by murine and primate studies, Osakada et al. used extended culture with the addition of retinoic acid and taurine to the culture medium of hESCs. Crx positive photoreceptor precursors increased from 11.3% of total cells on day 120 to 19.6% of total cells by day 170. This was mirrored by an increase in cells with a mature photoreceptor phenotype. Rhodopsin positive rod photoreceptors increased from 5.1% on day 150 to 8.5% of total cells on day 200. The protocol also generated cones with red/green opsin making 8.9 of total cells and blue opsin 9.4% of total cells (Osakada et al., 2008). More recently, Mellough et al. described a more complex protocol which investigated combinations of factors used by previous published protocols. Using flow cytometry, markers for mature photoreceptors peaked at day 45. However, this declined rapidly by day 60 with almost complete loss of rhodopsin (Mellough et al., 2012). The findings are different from other groups who found increases in photoreceptor markers with time (Osakada et al., 2008; Phillips et al., 2012), however, they suggest that photoreceptor differentiation can be accelerated. HESC studies have since been confirmed using hiPSC lines. Lamba et al. (2010) demonstrated similar results with hiPSC differentiation. The percentage of cells expressing markers of mature photoreceptors was still less than 1% of total cells after two months of culture. Similarly Osakada et al. found similar efficiency with disease and control hiPSCs (Jin et al., 2011; Osakada et al., 2009).

Table 5
Summary of characteristics of RPE verification.

Feature	Markers
Morphology (light microscopy)	Pigmentation, polygonal, monolayer
Morphology (transmission electron microscopy)	Apical microvilli, intracellular pigment granules, tight-junctional complexes across and basement membrane formation
Loss of pluripotency markers	OCT3/4, SOX2, NANOG
Non-specific RPE markers	MITF, SILV, PAX6, OTX2, Claudin-10
RPE specific markers	Phagocytosis: MERTK, FAK Pigmentation: Tyrosinase Growth factors: Pigment epithelium derived growth factor Visual cycle: RPE65, RLBP1, CRALBP
Polarity	Resting membrane potential Apical staining: Ezrin, Na^+/K^+ATPase Basal staining: BEST1
Phagocytosis	Photoreceptor outer segment phagocytosis assay
Tight junction formation	ZO-1 staining, transepithelial resistance >150 Ohms per cm^2

Table 6
Markers of different stages of retinal cell differentiation.

Stage	Markers
Pluripotent cell	OCT4, SOX2, TRA-1-60, NANOG
Epiblast	FGF5+
Primitive anterior neuronal	PAX6, OTX2
Anterior neuroepithelial	PAX6, OTX2, SOX1
Eye field	Rx, SIX3, SIX6, LHX2
Optic vesicle	PAX6, MITF, CHX10
RPE progenitor	PAX6, MITF
Neuroretinal progenitor	PAX6, CHX10
Photoreceptor	RECOVERIN, OPSIN, CRX
Mature RPE	BEST1, ZO-1, CRALBP, RPE65, PEDF, MITF, SILV, MerTK.

While it is clear that pluripotent stem cells produce neuroretinal progeny, identifying these cells in living cultures poses a challenge. Unlike RPE, plated clusters of differentiating neuroretinal cells are not readily discernible from non-retinal neural cell populations by direct visual or light microscopic inspection. Thus, a means to isolate NRPCs and/or their progeny from unwanted or unknown cell types in culture is a necessary step prior to transplantation in humans. Mechanically manipulated hESC and hiPSC cultures can be manipulated into generating populations of free-floating neural aggregates containing NRPCs, termed “OV structures” (Meyer et al., 2011). Since they share a number of properties with embryonic optic vesicles, they can be readily isolated based on their distinct appearance in culture and grown in bulk culture using a simple media formulation. Initially, these hiPSC-OVs consist of a nearly uniform population of proliferating CHX10+ NRPCs, but over time they produce cells expressing photoreceptor and other neuroretinal cell markers (Meyer et al., 2011; Phillips et al., 2012).

The future of hiPSC derived neuroretinal differentiation perhaps lies in the generation of a three-dimensional whole neuroretina which recapitulates retinal development *in vitro*. This was first demonstrated using murine ESCs (Eiraku et al., 2011) as has since been confirmed using hESCs by Nakano et al. (2012). Using this protocol hESCs were suspended in media containing WNT antagonist IWR1-endo, hedgehog smoothened agonist anti-apoptotic agent Y-27632 and 1% Matrigel. Addition of 10% FBS was found to markedly increase retinal progenitor formation with greater than 70% of cells found to be Rx positive by flow cytometric analysis. WNT agonist CHIR99021 was added temporarily following which a double walled optic cup structure self-forms from the invagination of the distal vesicle. Retinal photoreceptor maturation appeared to mirror foetal development in timing. However, the protocol still requires optimisation in order to produce photoreceptors with outer segments (Nakano et al., 2012). In addition, hiPSC-derived NRPCs also have the capacity to self-assemble into laminated retina-like structures (Phillips et al., 2012). When hiPSC-derived OVs were isolated and followed over time, a subset gave rise to multilayered retina-like structures (~16%). While the percentage of OVs that maintained laminated retinal architecture was modest, the use of a minimal culture system demonstrated the innate ability of hiPSCs for self-assembly. Long term neuroretinal culture was

maintained at 40% oxygen by Nakano et al. (2012). Interestingly, physiological oxygen levels have recently been shown to increase differentiation efficiency to both retinal progenitor and neuroretina (Bae et al., 2012; Garita-Hernandez et al., 2013). The difference may demonstrate that low oxygen tension plays a role in differentiation replicating *in vivo* conditions but that the maintenance of multi-layered organ structures *in vitro* requires a higher oxygen tension without a blood supply.

Several protocols have been reported for the generation of photoreceptors and other neuroretinal cell types from hiPSCs for cell replacement therapy. However, it is critical that these donor cells are functional for transplantation to be successful and for modelling of certain retinal diseases. Previous studies have suggested that neuroretinal cells derived from hiPSCs form chemical and electrical synapses in culture (Phillips et al., 2012), that these cells have an electrophysiological profile consistent with photoreceptors (Jin et al., 2011; Meyer et al., 2011), and express key components of the phototransduction cascade (Meyer et al., 2011). A recent study by Nasonkin et al. highlighted the critical function of RPE in outer segment formation *in vivo*. While Dnmt1 knockout in photoreceptors had no effect on photoreceptor outer segment genesis, RPE-specific Dnmt1 knockout led to disrupted RPE apicobasal polarity and pronounced defects in outer segment production (Nasonkin et al., 2013). Indeed, a study by McUsic et al. showed the importance of RPE-photoreceptor co-culture by culturing neonatal mouse photoreceptors in a microchannel scaffold, sandwiched on top of an RPE explant culture (McUsic et al., 2012). Using this culture method, photoreceptors formed disorganised outer segment material. Further studies are required to delineate methods for forming true photoreceptor outer segments *in vitro*. Outer segment formation will be critical for hiPSC disease modelling studies, as the majority of photoreceptor-based retinal disorders arise from mutations in proteins localised to the outer segment. *In vitro* outer segment formation is less critical for transplantation therapy, as transplanted photoreceptors may utilize the host environment and RPE to generate outer segments post-transplant.

3.3. Direct lineage conversion

The creation of hiPSCs demonstrated that defined factors can transform a terminally differentiated somatic cell to a pluripotent cell and stabilise a new epigenetic framework. A disadvantage of hiPSC based reprogramming is that somatic cells require transformation to a developmental 'ground state' before redifferentiation to the cell type of interest. Building on this work, it was proposed that terminally differentiated cells could be directly reprogrammed to other terminally differentiated cells without the need for a pluripotent intermediary. This may provide several advantages for cell therapy as it allows a means of deriving retinal cells without the associated difficulties in differentiation and maintenance of hiPSCs. This may be of especial benefit in protocols which currently have lengthy differentiation protocols such as those used in hiPSC-derived neuroretina. This process has been variously called direct lineage conversion, direct conversion or direct reprogramming. Work by Davis et al. (1987) demonstrated the introduction of a single transcription factor *MyoD* was able to convert mouse fibroblasts to myocytes. This suggested that there were master transcription factors that could regulate many genes to facilitate conversion in a transcriptional network. There are now several papers demonstrating that it is possible to alter cells from one somatic phenotype to another by introducing relevant transcription factors (Ieda et al., 2010; Szabo et al., 2010; Vierbuchen et al., 2010). Recently, an attempt was made to derive retinal cells using direct conversion from human fibroblasts. Zhang et al. developed a Best1: GFP reporter system to investigate the role of eight candidate transcription factors in direct conversion of fibroblasts to RPE. It was found that c-Myc, Mitf, Otx2, Rax, and Crx were essential for successful conversion to RPE-like cells. Daughter cells showed RPE-like expression profiles and stained for several RPE markers, but no functional analysis was provided (Zhang et al., 2013). Detailed verification will be important to show whether this technique is viable for the production of clinically relevant retinal cells.

Although direct lineage conversion techniques are more efficient than reprogramming to pluripotency, at present a maximum of one daughter cell forms from each donor cell. Therefore large quantities of donor cells would be needed to generate required cellular material. This issue could be resolved by reprogramming to a progenitor stage with subsequent more limited expansion when compared with hiPSCs. Recently, advancements have been made to realise this goal that could have implications for ophthalmic research. Using nine transcription factors murine mesodermal cells were converted to induced neural precursors. The precursor was both expandable and could be differentiated to the three neural lineages (Sheng et al., 2012). Other groups have now published protocols using as little as two factors to produce an expandable neuronal precursor with conversion varying from three days to one month with murine cells (Han et al., 2012; Lujan et al., 2012; Thier et al., 2012). Presently, no group has been able to demonstrate direct conversion to a retinal precursor.

Another barrier to the development of direct lineage reprogramming protocols is the requirement for complex transcriptome and bioinformatic analysis in order to identify potential transcriptional targets. This is likely to become easier as bioinformatics becomes more efficient. However, much remains to be learnt about the most effective way to use this evolving methodology in retinal research.

4. Clinical applications of hiPSC derived retinal cells

4.1. Disease modelling

As robust protocols have been established for the efficient, reliable generation of hiPSC-derived retinal cells, there has been an increased focus on developing clinical applications for retinal disease treatment (Fig. 5). The major attraction of hiPSC-based disease modelling is the potential to differentiate phenotype-relevant cell types that would enable the investigation of disease mechanisms in the context of specialised cellular physiology. In the routine context, functional human retinal cells are difficult to obtain and maintain. Consequently, this has led to the adoption of immortalised cell lines. However, these cellular systems are removed from the physiology of the post-mitotic, specialised cell types that are affected in human disorders, confounding inferences that can be gained about disease processes. HiPSC derived retinal cells are able to recapitulate key physiological and functional *in vivo* characteristics. This highlights the utility of hiPSC derived retinal cells in retinal disease modelling.

Recently, several papers have been published describing disease modelling using hiPSC-retinal cells. For example, a clinically relevant disease phenotype for Best vitelliform macular dystrophy (BVMD) was observed using hiPSCs from affected patients when compared to those obtained from unaffected siblings. BVMD is caused by a defect in the RPE gene *BEST1*, which results in the subretinal accumulation of photoreceptor waste products (lipofuscin) and fluid, leading to secondary photoreceptor death and central vision loss. Using physiological stressors, BVMD hiPSC-RPE showed evidence of a similar functional defect on a cellular level *in vitro*. Further analysis of BVMD and paired control hiPSC-RPE

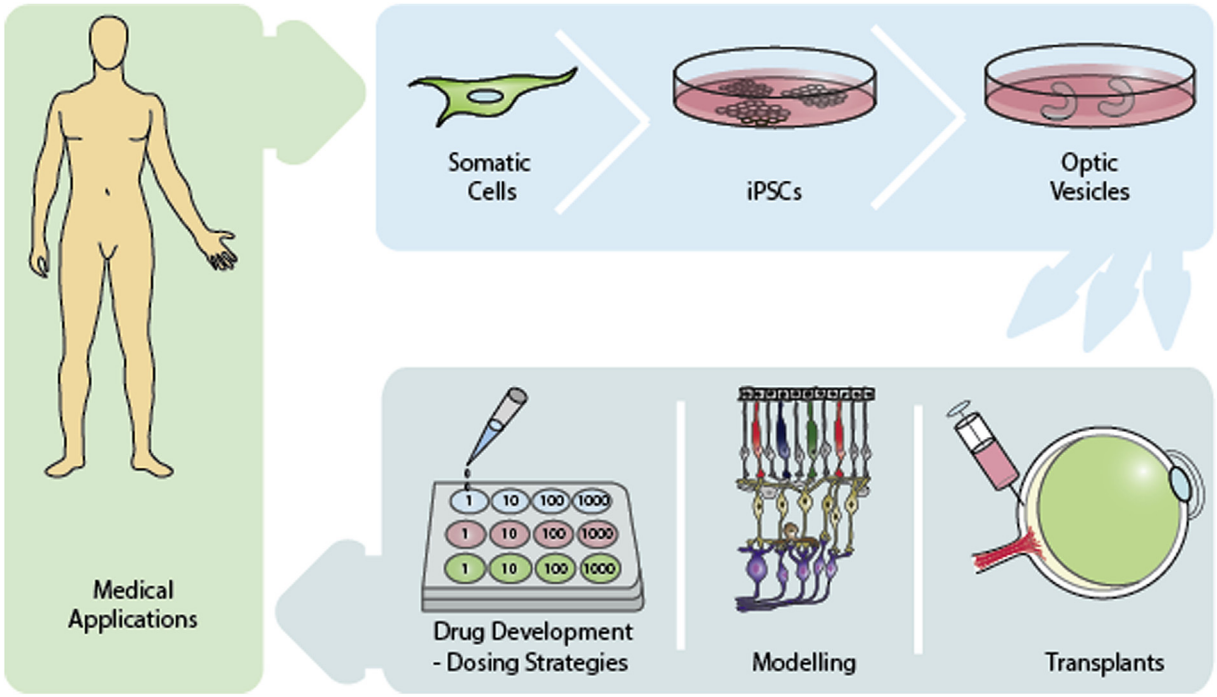

Figure 5. Diagram summarising potential clinical applications of retinal cells derived from hiPSCs.

cultures and comparison to a foetal human RPE overexpression system suggested a role for intracellular calcium regulation and oxidative stress in the mechanism of disease. Similarly disease relevant biomarkers were discovered in a neuroretinal model for retinitis pigmentosa using hiPSC-rod photoreceptors from cases and controls. The model showed increased expression of markers for oxidative and endoplasmic reticulum stress (Jin et al., 2011). Both of these studies highlight the potential of RPE and neuroretinal cells derived from hiPSCs to help identify pathophysiological pathways for targeted development of therapies.

The importance of modelling using human retinal cells rather than animal models or more easily accessible tissue was recently demonstrated by Tucker et al. who derived hiPSCs from a patient with sporadic RP. HiPSC retinal precursors were used to verify the pathogenicity of a homozygous Alu insertion into the causal gene uncovered by exome sequencing (Tucker et al., 2011). In this elegant study, the authors found that the insertion of the Alu sequence into exon 9 of the patient's male germ cell-associated kinase (*MAK*) gene prevented the expression of a splice variant of *MAK* that is normally expressed only in retinal precursors. In this case, hiPSCs offered an efficient means of identifying the disease mechanism by enabling the *ex vivo* study of patient-specific retinal cells.

Notwithstanding the value of studying isolated single cell types, it is clear that accurate modelling of disease processes requires development of more complex 3D systems that also incorporate other relevant cell types. One such platform is the self forming eye cup which was initially demonstrated using mouse embryonic stem (ES) cells (Eiraku et al., 2011) and later using human ES cells (Nakano et al., 2012) and hiPSCs (Phillips et al., 2012). The mouse and human ES cultures were produced in differentiation media supplemented with matrigel in order to provide extra cellular matrix. The resultant optic cup was multilayered and consisted of relevant retinal cell types including photoreceptors, ganglion cells and interneuron precursors. This method has the potential for allowing purification and propagation of specific retinal cells for use in isolation. More importantly, a three dimensional *in vitro* structure replicates the corresponding *in vivo* structure remarkably well, enabling the study of non-cell autonomous diseases that result from complex interactions of different retinal cell types.

4.2. Drug screening

Ultimately, hiPSC-based *in vitro* disease models could be used to perform compound screening, predictive toxicology and to evaluate new therapeutics. All of these screens would require assays that can be adapted for high throughput platforms. Such assays will need to be multiplexed to simultaneously assess the proliferation, morphology, differentiation, viability and function of specialised cell types that would enable the monitoring of disease-relevant phenotypes. In 2009, the first proof-of-principle hiPSC study was published that combined a demonstration of *in vitro* disease phenotypes and screening for candidate drugs using familial dysautonomia (FD) hiPSCs (Lee et al., 2009).

To date, there have been a limited number of publications looking at the effects of drugs on hiPSC-derived retinal cells. Meyer et al. (2011) reported the restoration of OAT enzyme activity in gyrate atrophy hiPSC-RPE following vitamin B6 treatment. Although B6 supplementation is a known treatment for gyrate atrophy the donor patient had been initially deemed unresponsive to vitamin B6 following surrogate clinical tests. The identification of B6 response in hiPSC-RPE suggested that systemic treatment in the patient would yield a beneficial clinical response. Therefore, this study underscored the potential importance of using custom hiPSCs to test the actual cell types targeted by a disease in order to more accurately assess drug efficacy. Similar studies in cardiology have led to drug testing that carries implications for dosing in humans and similar studies in the retina may be equally beneficial (Moretti et al., 2010).

HiPSC lines were created from patients with mutations in genes associated with RP, including *RP1, PRPH2, RHO*, and *RP9* (Jin et al., 2011). In this study, the authors noted death of RHODOPSIN-positive cells in all disease lines between days 120 and 150 of culture. However, treatment with α-tocopherol led to a statistically

significant preservation of RHO-positive cells only in retinal cultures carrying the RP9 mutation. Therefore, this study provided proof of principle that hiPSC technology is useful in screening for drug responses across numerous related, yet genetically distinct retinal diseases. This application is particularly important for genetically and phenotypically heterogeneous disorders like RP, and could ultimately help to narrow the disease targets for experimental drugs and hence inform clinical trial design. HiPSC technology offers an unlimited source of material for automated, high throughput drug screening systems to screen libraries of drug compounds. Ultimately, this will enhance drug discovery whilst concurrent toxicology studies will shorten the steps to phase 1clinical trial.

4.3. Limitations of the hiPSC platform: planning for disease modelling and toxicology studies

The first wave of 'proof-of-principle' studies has clearly demonstrated the utility of hiPSC-based disease models. However, they have also underscored a number of issues, both inherent to the reprogramming technologies and related to the downstream applications, which are yet to be resolved. Several molecular differences between hESCs and hiPSCs have now been established. Accumulating evidence suggests that most of these differences can result from differences in genetic background, passage number, viral integration, derivation conditions, clonal variations and source of primary cells used for reprogramming.

In this section we highlight key issues that need to be considered prior to planning of human pluripotent stem cell based experiments.

Pitfalls of hiPSC-based disease modelling include:

I. Genetic and epigenetic differences due to reprogramming and culture conditions (Fig. 6)
   a. Copy number variations and mutations arising from reprogramming and extended periods in culture
   b. Epigenetic differences: incomplete demethylation and remethylation in hiPSCs, aberrant X-chromosome inactivation or imprinting, repeat instability

II. Efficient and scalable derivation of enriched, phenotype-relevant cell types

III. Development of protocols that mirror *in vivo* cellular complexity
   a. Generation of functional, adult-like target cells as opposed to more immature cell types

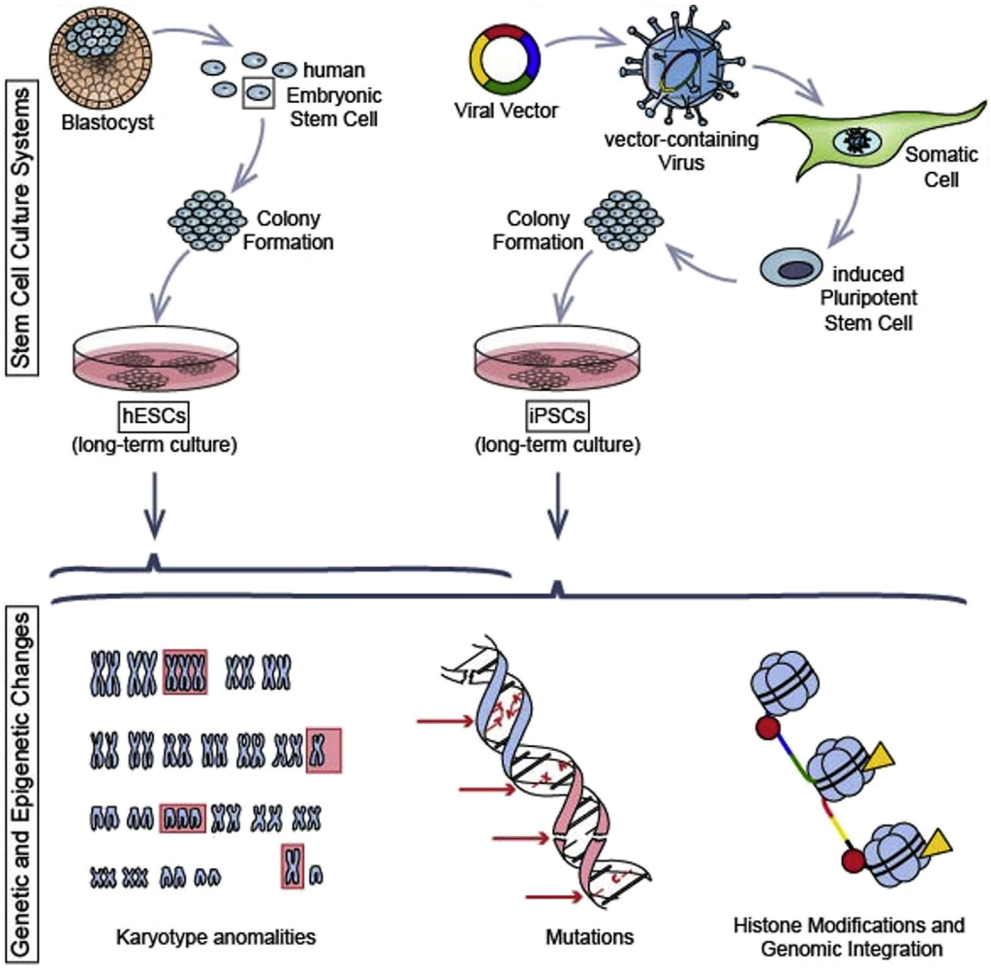

Fig. 6. Diagram summarising the similarities and differences between hESC and hiPSC cultures, together with the pitfalls induced by long term culture. HESCs are isolated from the inner cell mass of the developing blastocyst. Cells form rapidly dividing, flat, densely packed colonies containing cells with a high nuclear to cytoplasmic ratio. HiPSCs are created by the introduction of specific reprogramming factors into human somatic cells using various methods including viral vectors. HiPSCs share properties with hESCs. Both cell types require regular passaging. With prolonged culture and high passage number the risk for chromosomal aberrations and genetic mutation increase. HiPSCs have an additional risk of harbouring an abnormal karyotype, copy number variation and point mutation following the somatic cell reprogramming. Furthermore, hiPSCs can retain a memory of the donor tissue. Epigenetically, cells can exhibit DNA methylation defects and aberrant histone modifications. They can also have variable X chromosome inactivation.

b. Platforms that incorporate non-cell autonomous disease processes into the experimental setup

IV. Identification of disease-relevant and informative phenotypes that are technically robust, reproducible and amenable to high-throughput and high-content approaches

4.3.1. Epigenetics

Resetting of somatic cellular identity to a pluripotent state requires genome-wide epigenetic alterations that can be imperfect due to the limitations inherent in reprogramming methodologies. A number of studies have shown that hiPSCs can partially retain the epigenetic memory of the primary cells from which they originate, ultimately affecting the functional potential of these cells (Bar-Nur et al., 2011; Kim et al., 2010). In a comparative study of transcription-factor-based reprogramming of murine blood cells and fibroblasts it was demonstrated that both hiPSCs harboured differentially methylated DNA regions (DMRs) that correlated with their respective cell of origin (Kim et al., 2010), for example RPE derived hiPSCs showed a greater propensity to differentiate to RPE than hiPSCs derived from other lineages. Blood-hiPSCs also displayed a higher efficiency for haematopoetic differentiation potential compared to fibroblast-hiPSCs in directed-differentiation experiments suggesting that incomplete erasure of the epigenetic memory of the donor cell can limit the resulting pluripotent cells' propensity for differentiation (Hu et al., 2010). Epigenetic memory can be investigated using methylation studies.

A number of studies have already demonstrated that X-reactivation does not readily occur during derivation of human hiPSCs (Pomp et al., 2011; Tchieu et al., 2010). Moreover, the inactive X-chromosome in hiPSCs can also lose epigenetic silencing during extended culture, resulting in erosion of dosage-compensation (Mekhoubad et al., 2012). X-chromosome silencing can be checked by assessing *XIST* expression and histone H3-K27 trimethylated foci. Preferably valid cell lines can be taken once X chromosome silencing has been confirmed.

Failure of transcriptional activation of epigenetically silenced regions including the inactive X-chromosome upon reprogramming can impact on the utility of hiPSCs in modelling X-linked diseases. In Fragile X for example, the disease results from the epigenetic silencing of the Fragile X Mental Retardation gene (*FMR1*) during development due to a triplet-expansion in its untranslated region. In one study, hiPSCs were successfully derived from somatic cells of Fragile X patients but the resulting hiPSCs failed to activate the transcription of *FMR1* (Urbach et al., 2010). Therefore disease mechanisms and comprehensive controls need to be kept in mind due to potential epigenetic differences in hiPSCs which may be relevant for X-linked ophthalmic diseases such as X-linked retinitis pigmentosa.

4.3.2. Genetics and chromosomal anomalies

Another source of variation in hiPSC-based disease models are the genetic changes acquired during reprogramming and prolonged culture. Stem cell populations propagated *in vitro* accumulate chromosomal aberrations that include copy number variations, deletions in tumour suppressor genes and amplifications associated with oncogenes (Ben-David et al., 2011; Laurent et al., 2011). Long-term genetic stability of hiPSCs is also a particular concern in disorders with underlying repeat expansions such as Huntington's disease, Friedreich's ataxia (FRDA) and Fragile X syndrome (Mattis et al., 2012; Ku et al., 2010; Sheridan et al., 2011). In studying these models checkpoints against repeat instability should be incorporated early into the experimental design, paying particular attention to the specific mechanisms of repeat instability, as well as minimizing the time in culture. Checkpoints include genotyping regularly and comparing with baseline, good tissue culture and potentially using hypoxic conditions for maintenance.

The first generation of hiPSCs was created by the expression of reprogramming factors via integrative viral systems that inevitably resulted in the genomic insertion of the transgenes. These approaches not only carry the risk of insertional mutagenesis but also include the risk of incomplete proviral silencing that can increase clonal variation. In fact, hiPSC clones generated at a later stage following removal of transgenes harbour fewer genomic differences, lending support to the idea that integration of reprogramming factors can be a source of significant variation (Soldner et al., 2009). As discussed above, in order to remove this risk, newer non-integrative systems should be used for hiPSC generation.

4.3.3. Scale versus purity: limitations of directed differentiation

Although hiPSCs can theoretically give rise to any somatic cell type, high purity and sufficient scale of production can only be achieved currently for a limited number of specialised cell types. Culture heterogeneity is particularly pronounced in neural lineages, such as the neuroretina where distinct neuronal subtypes originate from a common progenitor through a series of symmetric and asymmetric cell divisions that progressively lead to lineage restriction (Gotz and Huttner, 2005). Therefore, reliable and consistent derivation of disease-relevant cell types from hiPSCs will require a combination of strategies that include the fine-tuning of developmental morphogens to mimic signalling in early embryonic development, cell type sorting and enrichment to achieve high purity and yield as described above. Many of these points have already been discussed above in the RPE and photoreceptor differentiation sections.

4.3.4. Extrapolating from in vitro disease-correlates to in vivo diseases

Two further challenges remain in disease modelling using patient-derived hiPSCs. Firstly, the generation of disease-relevant cell types that reflect the functional maturity observed *in vivo* and, secondly, the identification of robust molecular and functional differences between diseased cell types compared to healthy controls. It is critical that the disease in question has an appropriate phenotype that can be manifested and assayed in a dish.

In vitro differentiation protocols in different cell lineages have led to the differentiation of more immature cell types. For instance, cardiomyocytes derived from hESCs and iPSCs were found to be phenotypically immature, with electrophysiological properties similar to that of foetal cardiomyocytes (Kuzmenkin et al., 2009; Peng et al., 2010). Looking at hiPSC-ERPE, an RNA expression microarray profile of hESC/hiPSC derived RPE cells, the profile was found to be similar to foetal RPE. HESC-RPE had 88 transcripts with a difference compared to foetal RPE and hiPSCs showed 224 significant transcript differences. Using hierarchical clustering to analyse the data, hESC-RPE expression profiles clustered with foetal RPE whereas hiPSC-RPE was found in the next hierarchical cluster (Liao et al., 2010). It was worth noting that the cells in this study were investigated from as early as 2 months of culture. The time point of cell selection is important when investigating hiPSC-RPE phenotype as cells obtain a more mature phenotype with time in culture (Vaajasaari et al., 2011). HiPSC-RPE perform many of the functions associated with mature adult RPE including monolayer formation with tight junctions, production of good transepithelial resistance, polarized secretion of growth factors, appropriate fluid flux and ATP induced calcium response (Kokkinaki et al., 2011). Indeed hiPSC-RPE performed photoreceptor outer segment phagocytosis better than foetal RPE. Taken together these

assessments suggest the value of using hiPSC-RPE in modelling adult RPE disease.

Regarding hiPSC-derived photoreceptor like cells, there continues to be a need to further optimise differentiation protocols. Full adult photoreceptor morphology eludes current differentiation protocols. This is highlighted by the lack of production of outer segments by cultured photoreceptors. Additionally, the general scarcity of rods in differentiating human pluripotent stem cell cultures remains a significant barrier to retinal disease modelling. However, this is likely that some of the current difficulties can be rectified by the introduction of co-culture protocols which more closely resemble *in vivo* conditions (Nasonkin et al., 2013). Interestingly, some photoreceptor-expressed RP mutations that target components of the phototransduction cascade may be conducive to hiPSC modelling. Meyer et al. (2011) showed that, in addition to expressing key phototransduction genes, human pluripotent stem cell-derived photoreceptor-like cells exhibited a change in membrane potential in response to exogenous 8-Br-cGMP, which mimics the switch from a light-adapted to a dark-adapted state. Thus, opportunities exist to examine the functional effects of photoreceptor-specific mutations using hiPSCs, as long as the limitations of the culture system are kept in mind. Also, the immaturity of differentiated cell types may not always be a limiting factor if a developmental disease is investigated (Marchetto et al., 2010), or the underlying disease process is detectable regardless of model cell immaturity such as a channelopathy (Itzhaki et al., 2011).

Lack of knowledge of the specific cell types affected in a disease or situations where the end stage disease in patients is markedly different from early stage events can each further complicate the disease modelling readouts. *In vitro* disease modelling can become particularly challenging where more than one cell type contributes to the manifestation of the disorder. One such case has been demonstrated for amyotrophic lateral sclerosis (ALS) with *SOD1* mutations where disease-onset was shown to be inherent to motor neurones whereas neurodegeneration was driven non-cell autonomously by astrocytes (Di Giorgio et al., 2007). Similarly co-culture techniques for retinal diseases such as Usher 1B syndrome may clarify whether the disease results from photoreceptor defects alone or from aberrations in the photoreceptor-RPE complex (Williams and Lopes, 2011). Modelling of complex diseases will eventually require taking into consideration the environment within which the vulnerable, specialised cells reside and designing experimental setups that utilize multiple enriched cell types from hiPSCs to model non-cell autonomous interactions. This can potentially be investigated with co-culture experiments.

Determining robust *in vitro* disease-associated phenotypes will become increasingly important for drug discovery and screening studies. Culture heterogeneity and dynamics can mask subtle differences, necessitating the adoption of sensitive methods such as real-time single-cell longitudinal survival analysis to demonstrate differences (Bilican et al., 2013). There is usually a balance between the speed, sensitivity and scaling-up of assays used to assess cellular phenotypes. It may be necessary to identify surrogate markers of disease-phenotypes, for example in cases where the main disease-relevant readout is a biochemical assay that is not readily adaptable to a high-throughput format. It is therefore imperative to consider at the early experimental design stage of hiPSC-based *in vitro* disease models if and how the physiological phenotypes would manifest in the cells that are obtained from directed differentiation, how such differences would be measured and how many controls are necessary to document *in vitro* disease-correlates given natural variations in phenotypes.

4.4. Cell transplantation

The central nervous system (CNS), and the retina in particular, is an appealing target for cell replacement strategies due to poor ability of these tissues to self-repair. Additionally, despite the multitude of clinically identified neurodegenerative disorders there is currently a dearth of therapeutic options. The eye has several advantages over other CNS structures as a target for cell replacement strategies. These include surgical accessibility, clearly understood anatomy and physiology, and amenability to imaging and functional monitoring. In addition, the eye is a paired structure that is relatively isolated from the rest of the body, which reduces the impact of untoward systemic adverse effects of treatment and allows for comparison to the contralateral eye.

4.4.1. Retinal pigment epithelium

At present, all retinal cell types can be differentiated from both hESCs and hiPSCs (Rowland et al., 2012). However, successful cell replacement does not hinge on the ability to produce the target cell alone. The initial hurdle is to produce and isolate sufficient quantities of the desired donor cell type to implant into the eye. RPE appears a model cell in this regard. Extended monolayers of RPE can be isolated and transferred to a variety of substrates (Buchholz et al., 2009; Carr et al., 2009; Meyer et al., 2011; Meyer et al., 2009; Singh et al., 2013). In addition, RPE transplantation poses fewer challenges than neuroretinal cell transplantation, as the former cell type assumes a relatively simple monolayer structure and does not require synaptic integration. Consequently, RPE replacement using pluripotent stem cells has progressed relatively rapidly. Some information regarding pluripotent cell derived RPE transplantation has come from a multicentre trial run by Advanced Cell Technologies for the treatment of dry macular degeneration and Stargardt macular dystrophy (Schwartz et al., 2012). In these studies, a near pure population of RPE was obtained from hESCs maintained under good manufacturing practice (GMP) conditions and injected subretinally. Although results from this safety trial were preliminary, no untoward effects were seen in treated patients. Similarly, hiPSC-RPE has recently gained clinical trial authority approval to be used as the basis for patient treatment (Cyranoski, 2013).

HiPSC-derived RPE cell transplantation data are limited to animal models at present. Li et al. injected dissociated suspensions of hiPSC-RPE into the subretinal space of the RPE65 mouse model and showed some integration with the host RPE, as well as a modest improvement of visual function as measured by electroretinogram (ERG) (Li et al., 2012). Meanwhile Carr et al. (2009) showed that injection of dissociated hiPSC-RPE into the subretinal space of Royal College of Surgeons (RCS) rats, a model of secondary photoreceptor degeneration caused by a primary RPE defect, resulted in long-term preservation of visual function documented by optokinetic head-tracking. Similar results have been achieved in the RCS rat model using a wide variety of donor cell types, likely as a result of non-specific neuroprotective effects (Idelson et al., 2009; Inoue et al., 2007; McGill et al., 2007; Wang et al., 2008). However, the fact that some clusters of transplanted hiPSC-RPE displayed intracellular RHO staining suggested an ability to phagocytose photoreceptor outer segments *in vivo* (Carr et al., 2009), a critical function that is crippled by the *Mertk* mutation in RCS rats.

In addition to phagocytosing outer segments, hiPSC-RPE monolayers cultured *in vitro* exhibit a number of other important physiological properties, including formation of tight junctions, polarized secretion of growth factors, and stimulus-induced mobilization of intracellular calcium (Buchholz et al., 2009; Kokkinaki et al., 2011; Meyer et al., 2011; Singh et al., 2013). In contrast, bolus-injected RPE have not demonstrated such properties to date. Thus, to optimise the effects on host retinal function, it

may be preferable to manufacture and transplant intact hiPSC-RPE sheets on scaffolds. This strategy may also enhance donor RPE survival, since natural or synthetic, bioengineered scaffolds could provide a healthier substrate than host Bruch's membrane, particularly if the latter is damaged during the disease process (e.g. in age-related macular degeneration). Recently, Hu et al. described such a method to transplant hESC-derived RPE, grown on a 4 μm thick synthetic scaffold, into the subretinal space of RCS rats (Hu et al., 2012).

4.4.2. Photoreceptor

Although RPE is a popular target for cell replacement strategies, from a practical standpoint, most degenerative diseases involving the RPE that warrant stem cell-based therapies will have already caused extensive photoreceptor loss. Furthermore, the majority of inherited outer retinal degenerative dystrophies which are collectively referred to as retinitis pigmentosa (RP) and RP-like disorders, target photoreceptors, leaving the RPE largely unscathed. Therefore, it would be of great benefit to devise a means of replacing photoreceptors, with or without RPE, that have been lost due to disease or injury. As sources of donor neuroretinal cell types, human pluripotent stem cells have the distinct advantage of being both renewable and capable of producing true multipotent neuroretinal progenitor cells (NRPCs). Early during their maturation process, NRPCs are competent to yield all native classes of neuroretinal cells, including cones and rods, in a sequence that is highly conserved across vertebrate species.

While several papers have reported the generation of neuroretinal cells from hiPSCs, there are only rare reports of transplantation using these cells. In one study, Lamba et al. (2010) showed the migration of transplanted hiPSC-derived photoreceptors into the outer nuclear layer of a wild-type host mouse retina. Similar findings were reported using swine hiPSC-derived photoreceptors transplanted into the photoreceptor depleted swine retina (Zhou et al., 2011). In addition, transplantation of mouse hiPSC-derived photoreceptor precursors into the degenerative Rho −/− retina led to the integration of transplanted cells and limited restoration of a scotopic ERG response (Tucker et al., 2011).

Although information about transplantation of hiPSC-derived neuroretinal cells is scarce, insights gained from transplantation studies using other cell sources are invaluable for the advancement of cell replacement therapy using hiPSCs. Other cell or tissue sources that have been used for transplantation studies include foetal retina, foetal retina-derived photoreceptors, hESC-derived neuroretina, and photoreceptor precursors isolated from early postnatal retina. These studies have uncovered a number of hurdles that face cellular transplantation therapies, which include poor transplanted cell survival, migration and integration into the host retina. Previous studies transplanting mouse (West et al., 2012) or hESC-derived neuroretinal cells (Hambright et al., 2012) suggest that the transplanted bolus of cells often remain trapped in clusters within the subretinal space; however, results from Lamba et al. (2009) demonstrated integration of human ESC-derived neuroretinal cells and the presence of an ERG waveform in the transplanted *Crx* −/− mouse retina. Poor migration and integration of cells into the host retina could arise from either the intrinsic properties of the transplanted donor cells, from host retinal barriers, or more likely, a combination of both. Pioneering work by Robin Ali's group suggested that rod photoreceptor precursors must be isolated at a specific time in the developing mouse retina (~P4) if they are to migrate and integrate into the host retina (MacLaren et al., 2006), although a study from Gust and Reh proposed that adult photoreceptors may also possess these abilities (Gust and Reh, 2011). In future studies, hiPSC-derived photoreceptors must be transplanted at various stages of differentiation to determine the optimal developmental stage for transplantation. In addition, proper controls must be utilized to evaluate the effects of cell replacement. Endogenous reporters may be preferable to viral cell labelling to eliminate the possibility of viral particle carryover and potential mislabelling of host cells (West et al., 2012; Gonzalez-Cordero et al., 2013).

Pearson et al. (2012) published the most definitive report of vision restoration to date with cell replacement therapy. In this study they injected P4–P8 rod precursors into the *Gnat* −/− mouse, which lacks the rod α-transducin required for rod function. Following cell replacement, the authors showed morphological and synaptic integration of the transplanted cells, and functional scotopic vision rescue through a battery of tests. These tests included single cell electrophysiology which showed a transplanted rod photoreceptor response to light, optical intrinsic imaging to demonstrate relay of the visual signal to the visual cortex, and rescue of an optokinetic response and visually guided behaviour. Additionally, a recent study by Gonzalez-Cordero et al. suggested that 3D culture generated mouse ESC-derived photoreceptors improved integration (Gonzalez-Cordero et al., 2013), compared to the dearth of integration seen with 2D methods for photoreceptor production (West et al., 2012). Taken as a whole, these animal studies highlight the potential for cell based transplantation therapy. However, whether these results can be repeated using hiPSC-derived retinal progeny in human patients remains to be determined.

5. Future directions: transferring hiPSC-derived cell therapy to clinic, risks and challenges

In order to bring the clinical promise of hiPSCs to realisation a number of hurdles must first be overcome. Tumour formation is undoubtedly the most serious potential complication associated with cell replacement therapy from pluripotent stem cell sources. Great care must be taken prior to transplantation to assure that no pluripotent cells remain. Tucker et al. used the cell-surface pluripotency marker SSEA1to immunopan for undifferentiated pluripotent cells prior to the transplantation of mouse iPSC-derived neuroretinal cells (Tucker et al., 2011). Following two rounds of SSEA1+ cell depletion, the development of teratomas were eliminated at three weeks post-transplantation. While negative selection of pluripotent cells is one strategy to minimize tumour risk, an alternative approach is positive selection for post-mitotic photoreceptors. Studies using hiPSCs, mouse ESCs, or photoreceptor precursors obtained from early postnatal mouse retina have used various sorting methods to enrich for photoreceptors. However, most studies have sorted photoreceptors using transgenic fluorescent protein expression driven by the promoters of photoreceptor genes such as *IRBP, CRX*, or *NRL* (Lakowski et al., 2010; Lamba et al., 2010; Pearson et al., 2012; Pearson et al., 2010; West et al., 2010; West et al., 2008), or alternatively have sorted retinal progenitors earlier in development with RAX (West et al., 2012). While effective, the use of fluorescent transgenic markers is unlikely to be suitable for clinical studies. Sorting without reporter insertion could be potentially feasible with recently identified cell surface markers including CD73 alone (Eberle et al., 2011; Koso et al., 2009), CD73 plus CD24 (Lakowski et al., 2011), or lectins to enrich for photoreceptors (Balse et al., 2005; Mandai et al., 2010). Even so, it is currently unclear if these sorting methods would identify other non-retinal cell types in a mixed population of cells derived from pluripotent cell sources. For example, CD73 is expressed in numerous cell types throughout the body, and is also upregulated in cancerous cells (Zhang, 2010). For these photoreceptor selection methods to work with pluripotent cell sources, they must either: a) be tested rigorously for the selection of all other unwanted cell

types in the body, or, more realistically, b) must only be used on a starting population of enriched neuroretinal progenitors derived from hiPSCs. This can be achieved by using a method to enrich for optic-vesicle like structures, as discussed previously (Meyer et al., 2011).

The host retina may also create barriers that transplanted cells must cross, including the outer limiting membrane (OLM) and, not uncommonly, gliotic scars. The severity of impediment is dependent upon disease type, as well as disease progression. Differences in disease type and progression on cell transplantation outcome were systematically evaluated by Barber et al. (Barber et al., 2013). This study demonstrated the large degree of variability in gliosis and OLM impediment to cellular integration across six mouse models of retinal degeneration at different stages of degeneration. Previous studies transplanting photoreceptor precursors have attempted to circumvent the physical barriers by pharmacological treatment with the glial toxin, dl-alpha-aminoadipic acid (West et al., 2008), or RNAi knockdown of tight junction protein ZO-1 (Barber et al., 2013; Pearson et al., 2010). However, potential untoward effects of such treatments, such as disruption of the outer blood-retinal barrier, may preclude their use in humans. As such, clinically relevant methods of bypassing the OLM and glial scarring are still needed to optimise migration and integration of transplanted cells into the human retina.

The majority of transplantation studies thus far have focused on bolus injections of cells, regardless of the cell source. This may be the easiest method of cell delivery, but transplantation of RPE and perhaps photoreceptors as sheets on scaffolds or embedded in biocompatible matrices may be preferable (Diniz et al., 2013). Establishing polarity in transplanted cells is critical considering the unique apical and basal functions of both the RPE and photoreceptors. In addition, RDDs that arise from RPE defects will likely require replacement of both RPE and photoreceptors combined. However, building a combined RPE/photoreceptor scaffold for transplantation is challenging due in part to the naturally weak connections between photoreceptor outer segments and interdigitating RPE microvilli.

For hiPSC-derived cell transplantation therapy to become a reality, all phases of the generation of retinal cell types must be performed following current GMP guidelines. To begin with, hiPSCs must be generated using non-integrative approaches. This stage has been assisted by the recent development of a completely chemically defined and xeno-free method for derivation and maintenance of pluripotent stem cells (Chen et al., 2011). The media (E8) eliminates many of the reagents in previous pluripotent stem cell media formulations, including serum albumin, and can be used in combination with vitronectin coated surfaces for cell adhesion and growth, eliminating the need for MEF or Matrigel, sources of animal contaminants. Recent reports have identified methods for retinal differentiation of hiPSCs using xeno-free methods. The combination of new hiPSC derivation protocols with chemically defined differentiation protocols should enable the realisation cGMP compliant retinal cells for cell replacement (Sridhar et al., 2013; Tucker et al., 2013).

HiPSC cells used for autologous cell replacement may hold certain immunological advantages over generic hESC cells. However, these cells carry the same genetic mutations as the originator and are therefore susceptible to the same disease processes as the host retinal cells. Autologous hiPSC-derived cell transplantation for the treatment of genetic-based retinal disorders will first require correction of the underlying disease-causing mutation. A number of techniques are being used for editing genes in hiPSCs. The first of these technologies is zinc finger nucleases (ZFNs), an artificial DNA sequence specific enzyme that promotes DNA strand breaks at specific locations in the genome. The strand break and excision promote activation of normal cellular DNA repair processes including non homologous end joining and homologous repair. These can be used in combination with specifically designed plasmids used as a template for gene editing. The technology benefits from relative ease of design, but are expensive and may lead to off target gene editing. This technology has already been demonstrated in hiPSCs (Zou et al., 2009). Yusa et al. (2011) showed restoration of alpha-1 antitrypsin in alpha-1 antitrypsin deficient cells derived from a patient using ZFNs in combination with piggybacterial artificial chromosome (BAC) technology.

Recently, a BAC-based vector was used to correct the underlying disease-causing mutation in the gene encoding ornithine-δ-aminotransferase (*OAT*), restoring the function of this critical enzyme in hiPSC-RPE (Howden et al., 2011). A similar but cheaper technique to ZFNs uses transcription activator-like effector nucleases (TALENs) (Cermak et al., 2011). This technique offers the benefits of gene editing with the ability to more easily create gene specific nucleases. Currently, the costs of such patient specific cell development are prohibitive, but it may be possible to generate a bank of hiPSCs created from a limited number of homozygous HLA-haplotyped donors that would cover a large proportion of the population. For example, it has been calculated that 150 selected homozygous HLA-typed individuals would cover 93% of the population in the UK (Gundry et al., 2012). In the United States, a bank of only 100 hiPSCs would cover the majority of ethnicities (European American, Asian, Hispanic, and African America) (Gourraud et al., 2012). However, partial HLA haplotype matching may be insufficient to stave off immune rejection. In mice, expression of even minor histocompatibility antigens was enough to elicit acute rejection of cells differentiated from mouse ES cells (Robertson et al., 2007). Even so, the inherent immune privilege of ES cell-derived tissues facilitated the induction of transplantation tolerance via minimal host conditioning, leading to long term donor cell survival (Robertson et al., 2007). Should HLA-matched and/or autologous hiPSCs exhibit an immunological advantage in humans, it will need to be determined whether the benefit(s) warrant the additional labour and expense required for their creation (Okita et al., 2011; Zhao et al., 2011).

6. Conclusion

HiPSC technology was developed following new understanding in different fields of biological science including nuclear transfer and gene regulation through key transcription factors. Recent recognition of the importance of this series of breakthroughs was acknowledged with the awarding of the 2012 Nobel prizes for medicine and physiology to Sir John Gurdon and Shinya Yamanaka. As with any new platform the ideal use of the new technology has taken time to be understood. The alignment of clinical research with basic scientific understanding is now beginning to show considerable promise in bringing clinical translation as has been demonstrated in this article. At present, the need for dedicated personnel to reprogram, select and maintain initial colonies of hiPSCs requires that most research is restricted to large academic institutions which have a central core stem cell facility. As reprogramming, differentiation and cell characterisation protocols continue to improve it is likely that stem cell technology will become more widely accessible. A further step change that is likely to make translation easier is the development of industry and biotechnology collaboration in order to develop large-scale stem cell production. Together these developments are likely to increase the availability of hiPSC based technology for the use in investigative and translational studies in the future. In recent years major progress has been made in bringing iPS applications to clinical trial and whilst many challenges remain, the future direction of iPS

development offers the realistic hope of reducing progression and possibly improving visual function for patients with currently untreatable retinal diseases.

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